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Effects of Low, Subchronic Exposure of 2,4-Dichlorophenoxyacetic Acid (2,4-D) and Commercial 2,4-D Formulations on Early Life Stages of Fathead Minnows (*Pimephales promelas*)

Gavin K. Dehnert,[a,*] Mariella B. Freitas,[b] Zachary A. DeQuattro,[c] Terence Barry,[d] and William H. Karasov[c]

[a]Department of Integrative Biology, University of Wisconsin-Madison, Madison, Wisconsin, USA
[b]Department of Animal Biology, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil
[c]Department of Forest and Wildlife Ecology, University of Wisconsin–Madison, Madison, Wisconsin, USA
[d]Department of Animal Science, University of Wisconsin–Madison, Madison, Wisconsin, USA

Abstract: Aquatic herbicides are commonly used to control a wide variety of algae and plants, but they also have the potential to contaminate and affect nontarget organisms. However, the impacts of low-level 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide exposure on larval fish are not well understood. We conducted a series of experiments to determine the effects of low concentrations (0.05, 0.50, and 2.00 ppm) of 2 commercial 2,4-D amine salt herbicide formulations (Weedestroy® AM40 [WAM40] and DMA® 4 IVM [DMA4]) and pure 2,4-D on the development and survival of fathead minnows (*Pimephales promelas*) at various life cycle stages. Larval survival (30 d post hatch [dph]) was decreased following exposure of eggs and larvae to pure 2,4-D (0.50 ppm; $p \leq 0.001$), as well as to WAM40 (0.50 and 2.00 ppm; $p \leq 0.001$, $p \leq 0.001$) and DMA4 (0.50 and 2.00 ppm; $p \leq 0.001$, $p \leq 0.001$). The results also narrowed the critical window of exposure for effects on survival to the period between fertilization and 14 dph. Development was not negatively altered by any of the compounds tested, although the commercial formulations increased larval total length and mass at 2.00 ppm. Altogether, the results indicate that the use of 2,4-D herbicides for weed control in aquatic ecosystems at current recommended concentrations (<2 ppm whole lake; <4 ppm spot treatment) could present risks to fathead minnow larval survival. *Environ Toxicol Chem* 2018;37:2550–2559. © 2018 SETAC

Keywords: Contaminants; Developmental toxicity; 2,4-Dichlorophenoxyacetic acid; Aquatic toxicology; Herbicide

INTRODUCTION

Aquatic herbicides have the potential to contaminate surface water and groundwater (Ritter 1990), affecting nontarget fish species in their natural habitats (Barbieri 2009; Menezes et al. 2015; DeQuattro and Karasov 2016; Ruiz de Arcaute et al. 2016). The active ingredient 2,4-dichlorophenoxyacetic acid (2,4-D) is contained in many systemic herbicides used worldwide for selective weed control in agriculture and for control of invasive plants in public and private aquatic systems (e.g., Eurasian milfoil). At low concentrations, 2,4-D mimics auxin, thereby promoting uncontrolled plant cell growth (elongation and cell division; Grossmann 2003; Song 2014). The active ingredient 2,4-D also causes abnormal growth in the vascular tissue, senescence, and plant death (US Environmental Protection Agency 2005; Song 2014). The US Environmental Protection Agency (USEPA) permits aquatic 2,4-D amine applications up to 4 ppm for spot treatments and up to 2 ppm for whole-lake treatments, with an allowance for follow-up treatment 21 d after the initial application (US Environmental Protection Agency 2005). The half-life of 2,4-D in water is highly variable but is stated as being 15 d in aerobic water systems (US Environmental Protection Agency 2005). The Wisconsin Department of Natural Resources reported finding concentrations above the irrigation water threshold (0.10 ppm) 93 d after a spot lake treatment using 2,4-D at 0.50 ppm (Nault et al. 2014). Persistence of 2,4-D herbicide formulations may pose a risk to aquatic organisms because nontarget organisms are likely being exposed for periods longer than anticipated by regulations. We chose to test a range of treatment concentrations at or below whole-lake treatment permit levels (i.e., 0.05, 0.50, and 2.00 ppm) to improve our understanding of

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* Address correspondence to dehnert2@wisc.edu
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prolonged (subchronic) low-level exposure to two 2,4-D herbicide amine formulations and the active ingredient, technical grade 2,4-D acid.

The USEPA classifies 2,4-D as slightly to moderately toxic. Previous acute toxicity trials using 2,4-D pure compound were performed on a variety of fish species, such as rainbow trout, bluegill, striped bass, banded killifish, white perch, eel, and carp (US Environmental Protection Agency 2005). No-observed-effect concentrations for pure 2,4-D were determined to be in the range of 14.2 to 63.4 ppm acid and amine form (US Environmental Protection Agency 2005). Most of this previous research, however, used either adult or juvenile stages, and tested the pure 2,4-D compound rather than actual herbicide formulations, which are often composed of some percentage of 2,4-D (i.e., active ingredient) and a percentage of other compounds thought to be inert (i.e., nonactive ingredient). In earlier studies, adult fathead minnows exposed to pure 2,4-D compound for 21 d showed no significant change in fish fertility, body weight, or length (Coady et al. 2013). In contrast, a recent study on juvenile silver catfish exposed for 90 d to a 2,4-D amine salt herbicide formulation, U 46® D-fluid, showed a decrease in growth parameters at 0.50 and 2.00 ppm (Menezes et al. 2015). Inert labeled ingredients in formulations can be toxic on their own, can increase the toxicity of the active ingredient, can increase exposure to the active ingredient, and/or can increase the toxicity of the formulation (Oakes and Pollak 1999, 2000; Cox and Surgan 2006; Pérez et al. 2011; Mesnage et al. 2014). To accurately predict the effect of 2,4-D herbicides on freshwater systems and effectively manage their use, studies must be conducted on the actual herbicide formulations and not just the active ingredient 2,4-D.

Early developing stages in aquatic organisms are typically at higher risk from toxicants than their adult counterparts (Lotufo and Fleeger 1997; Mácová et al. 2008; Fairchild et al. 2009; Mensah et al. 2011). Although early developmental stages often represent a more susceptible period for toxicant exposure (Laale and Lerner 1981; Von Westernhagen 1988; McKim 1995; Bentivegna and Piatkowski 1998; Oikari et al. 2002; Mohammed 2013), most studies on the effects of fish exposed to 2,4-D used either adult or juvenile life stages. To accurately predict the effect of 2,4-D herbicides, it is crucial to explore their impacts on the survival and development of fishes, especially during early development (embryo and larva stages), to understand their total impact.

A recent study conducted in our laboratory (DeQuattro and Karasov 2016) continuously exposed adult fathead minnows (4 wk) and their offspring (from fertilization to 30 d post hatch [dph]) to DMA® 4 IVM (DMA4). The results showed a significant decrease in larval survival at concentrations as low as 0.034 ppm. Therefore, we first wanted to investigate whether other 2,4-D herbicide formulations would show similar negative impacts on larval survival. Consequently, we ran the same experiment with another commonly used 2,4-D formulation, Weedestroy® AM40 (WAM40). Second, we explored whether there is a critical life-stage window of exposure for fathead minnows. Thus, we narrowed our exposure period to include fertilization to 30 dph for both formulations to determine whether this would cause the same negative impacts on larval survival. Finally, we wanted to determine whether these impacts following 2,4-D exposures were due to the active ingredient (pure 2,4-D) or to the formulations as a whole (DMA4, WAM40).

MATERIALS AND METHODS

Experimental guidelines

We completed a series of 3 experiments (Table 1). The purpose of experiment 1 was to determine whether the herbicide formulation WAM40 showed similar effects to the herbicide formulation DMA4, as previously described in DeQuattro and Karasov (2016). The purpose of experiment 2 was to narrow down the critical window of exposure and to determine whether larval survival was still negatively impacted if the parental generation was not exposed. Finally, the purpose of experiment 3 was to test whether impacts observed in experiment 2 were due to the active ingredient (pure 2,4-D) or to the herbicide formulations as a whole.

Chemicals

Pure technical grade 2,4-D acid (pure 2,4-D; purity >95%), WAM40 (46.8% 2,4-D), and DMA4; (46.3% 2,4-D) commercial 2,4-D amine liquid herbicides were purchased from Grainger Lab Supplies, Forestry Suppliers, and NewTech Bio, respectively. Concentrated stock solutions for each formulation were prepared such that their delivery to the dilution cells would result in target concentrations of 0.00 (control), 0.05, 0.50, and 2.00 ppm of 2,4-D at the tank level in our system (experiment 3: 0.00, 0.50, and 2.00 ppm). The stock solutions for the

TABLE 1: Summary of life stages and commercial 2,4-D formulations tested over all 3 experiments[a]

	Life stage at exposure		
Experiment identification (herbicide formulation)	Adult	Embryo	Larval
DeQuattro and Karasov, 2016 (DMA4)	X	X	X
Exp. 1 (WAM40)	X	X	X
Exp. 2 (WAM40 and DMA4)	—	X	X
Exp. 3 (pure 2,4-D, WAM40, and DMA4)	—	X	X

[a]X indicates exposure at a life stage, and — indicates unexposed at the life stage.
WAM40 = Weedestroy® AM40; DMA4 = DMA® 4 IVM; 2,4-D = 2,4-dichlorophenoxyacetic acid; Exp. = experiment.

exposure system were prepared in Pyrex glass by diluting parent herbicide or pure compound with distilled water. The resulting mixture was aliquoted to 1 L amber glass bottles. Stock solutions were prepared every 6 d, stored in the dark at 8 °C, and held for no more than 6 d. A new amber bottle for each treatment was placed into the exposure system daily.

Fathead minnow husbandry

Fathead minnows were used as a model aquatic vertebrate because they are native to North America, abundant in the Great Lakes ecosystem (USA/Canada) where these formulations are used, and are accepted as an excellent fish model given their conserved endocrinology (Ankley and Villeneuve 2006). Seven-month-old adult fathead minnows were obtained from the Wisconsin State Laboratory of Hygiene (WSLH; Madison, WI, USA). Fish were brought to the University of Wisconsin–Madison Water Science and Engineering Laboratory and maintained in 60-gallon tanks (~100 minnows/tank) as large sex-specific groups, in a flow-through system at 25 ± 1 °C under a 16:8-h light:dark photoperiod. Adult fish were fed frozen adult brine shrimp (Brine Shrimp Direct) ad libitum twice daily. Temperature was measured daily (25 ± 1 °C); dissolved oxygen (8 ± 0.5 mg/L), pH (6.8–7), hardness (250 ppm), and ammonia (nondetectable) were measured weekly. All exposures and laboratory practices using fathead minnows were reviewed and approved by The University of Wisconsin–Madison under RARC protocol A005702.

Exposure system and chemical analyses

The present study used a flow-through exposure system. Two 4-channel, peristaltic pumps (model 07523-90, Cole Parmer) delivered the control (distilled water) and three 2,4-D stock solutions through tubing (size L/S 13 Masterflex® Norprene®, Cole Parmer) to individual glass dilution chambers at a rate of 0.5 mL/min. Heated (25 ± 1 °C), carbon-filtered City of Madison (WI, USA) water was added to the dilution chambers at a flow rate of 1.5 L/min, which diluted the 2,4-D stock solutions to the target 2,4-D concentrations of 0.00, 0.05, 0.50, and 2.00 ppm of 2,4-D. The water then flowed from each dilution chamber to 6 replicate exposure tanks (10 L), with 12 tanks/treatment. The flow rate into each tank was 250 mL/min (1 tank turnover/h). Each dilution chamber and all 48 tanks (12 × 4 treatments) in the system were constantly aerated. For adult exposures, each exposure tank contained a 110- × 90- × 70-mm spawning tile made by cutting a polyvinyl chloride pipe in half lengthwise. For larval exposure, the exposure systems were the same as above except they did not contain a spawning tile.

Water samples for 2,4-D level analysis were taken before the bottle solutions were renewed for the day. Samples were immediately frozen and stored at –20 °C. Analysis was performed by the Wisconsin State Laboratory of Hygiene using a 2,4-D enzyme immunoassay (ELISA) kit (minimum detectable level = 1 ppb; Modern Water). Treatment groups were named by their nominal concentration of 2,4-D as measured by ELISA. For experiments 1 and 2, concentrations of 0.00, 0.05, 0.50, and 2.00 ppm of each 2,4-D formulation (WAM40 and DMA4) were confirmed by measuring samples from 4 randomly selected tanks from each treatment every week (Table 2). All 3 treatments differed from one another in 2,4-D concentration as expected ($F_{3,15} = 1603$, $p \leq 0.0001$; Supplemental Data, Figure S1). For experiment 3, target concentrations of 0.00, 0.50, and 2.00 ppm of each 2,4-D formulation (pure 2,4-D, WAM40, and DMA4) were confirmed by measuring samples from 4 tanks selected randomly from each treatment every week (Table 3). For experiment 3, all 3 treatment groups with 0.50 ppm were not significantly different in 2,4-D concentration ($F_{2,23} = 2.402$, $p > 0.05$), and all 3 treatment groups with 2.00 ppm were not significantly different in 2,4-D concentration ($F_{2,23} = 3.166$, $p > 0.05$). Target concentrations (ppm) will be referred to from here on. When referring to active ingredient 2,4-D technical grade, the term 2,4-D will be used, and when referring to herbicide formulations, their respective names will be used from here on.

Endpoints

At the end of all the experiments, animals were euthanized with mass-appropriate buffered concentrations of MS222. Animals were gently blotted with Kimwipes to remove excess fluid, and wet mass was determined using an Ohaus analytical balance with ±0.001 g precision. Total length of animals was determined using a digital Mitutoyo absolute digimatic digital caliper with ±0.01 mm precision. Length measurements were made from the tip of the longest jaw or end of the snout to the longest caudal lobe (Kahn et al. 2004). Survival at various time points was determined by visual counting.

TABLE 2: Measured 2,4-D concentrations for experiments 1 and 2[a]

Target 2,4-D concentration (ppm)	Measured 2,4-D concentration (ppm)
0.00	ND
0.05	0.041 ± 0.0013
0.50	0.39 ± 0.0091
2.00	1.95 ± 0.043

[a]Measured values are mean ± standard error of the mean ($n = 16$ samples for each treatment).
ND = nondetectable; 2,4-D = 2,4-dichlorophenoxyacetic acid.

TABLE 3: Measured 2,4-D concentrations for experiment 3[a]

Target 2,4-D concentration (ppm)	Measured 2,4-D concentration (ppm)
0.00	ND
0.50 WAM40	0.36 ± 0.045
0.50 DMA4	0.45 ± 0.059
0.50 Pure 2,4-D	0.29 ± 0.048
2.00 WAM40	2.17 ± 0.253
2.00 DMA4	2.58 ± 0.0646
2.00 Pure 2,4-D	2.02 ± 0.114

[a]Measured values are mean ± standard error of the mean ($n = 16$ samples for each treatment).
ND = nondetectable; 2,4-D = 2,4-dichlorophenoxyacetic acid; WAM40 = Weedestroy® AM40; DMA4 = DMA® 4 IVM.

Experiment 1: Exposed adults, eggs, and larvae (WAM40)

Adult exposure consisted of reproductive groups comprised of 2 adult females and 1 adult male that were assigned randomly to each treatment. After a 15 d pre-exposure period involving 96 such groups, the 44 reproductive groups that showed the most consistent daily spawning (1 spawn/3–7 d) and egg production (>50 eggs/spawn) were selected for the 28-d exposure phase of the assay. These groups were then allocated to 4 treatments (0.00, 0.05, 0.50, and 2.00 ppm; 1 reproductive group/tank; $n = 11$ tanks/treatment). One tank in each treatment was left empty to accommodate tiles with fertilized eggs to allow them to hatch under exposure. To prevent bias in egg production by treatment at the start of the exposure, the groups were assigned based on their pre-exposure fecundity rate, such that the mean egg production/female/day was balanced across treatments. For example, the 4 groups that showed the highest fecundity during the pre-exposure were randomly allocated among the 4 different treatments, and so on.

For egg and larvae exposure, fertilized eggs laid by exposed adults were collected as available during days 26 to 33 of adult exposure. Several spawns (~1000 eggs/treatment, minimum 6 tiles) in each treatment were collected and allocated within tanks according to their respective treatments (i.e., eggs laid by adults exposed to 0.00 ppm WAM40 were allocated to the 0.00-ppm treatment tanks, and so on). Subsequently, eggs developed and hatched under exposure. Tiles were removed after hatch and 2-d-old larvae were allocated to 12 tanks within their respective treatments ($n = 10$ larvae/tank). Larvae were raised to 30 dph in a 10-L tank. Larval survival per tank was determined weekly throughout the exposure. After 30 d of exposure, larval fish were euthanized, counted to determine larval survival ($n = 10$ tanks for 0.00 ppm; $n = 12$ tanks for 0.05, 0.50, and 2.00 ppm), measured for total length (±0.01 mm), and weighed for wet mass (±0.001 g).

Experiment 2: Unexposed adults and exposed eggs and larvae (WAM40 and DMA4)

Unexposed adult fish were housed (2 females to 1 male; $n = 30$ reproductive groups) in a flow-through tank with one spawning tile each. Within a 24-h period, 21 tiles, containing 50 to 200 eggs, were distributed into the 4 target concentrations with approximately the same number of eggs in each ($n = \sim 800$ eggs/treatment). The eggs developed and hatched under exposure to target concentrations of WAM40. The tiles were removed from the hatching tank when hatches were complete (at 5–7 d), and 2-d-old larvae were allocated to 12 tanks within their respective treatments ($n = 10$ larvae/tank). Larvae were raised to 30 dph in a 10-L tank. Larval survival/tank was determined weekly throughout the exposure. After 30 d of exposure, larval fish were euthanized, counted to determine larval survival ($n = 12$ for 0.00, 0.05, 0.50, and 2.00 ppm), measured for total length (±0.01 mm), and weighed for wet mass (±0.001 g). The same experiment was repeated using the herbicide formulation DMA4.

Experiment 3: Unexposed adults and exposed eggs and larvae (WAM40, DMA4, pure 2,4-D)

Unexposed adult fish were housed (2 females to 1 male; $n = 40$ reproductive groups) in a flow-through tank with one spawning tile each. Within a 24-h period, 30 tiles, containing 50 to 200 eggs, were distributed into the 4 target concentrations with approximately the same number of eggs in each ($n = \sim 800$ eggs/treatment). Eggs developed and hatched during exposure to target concentrations of pure 2,4-D, WAM40, and DMA4 (0.00, 0.50, and 2.00 ppm). Tiles were removed from the hatching tank when hatches were complete (after 5–7 d), and 2-d-old larvae were allocated to 12 tanks within their respective treatments ($n = 15$ larvae/tank). Larvae were raised to 30 dph in a 10-L tank. Larval survival/tank was determined weekly throughout the exposure. After 30 d of exposure, larval fish were euthanized, counted to determine larval survival ($n = 22$ for 0.00 ppm; $n = 12$ for 0.50 ppm pure 2,4-D, WAM40, and DMA4; and 2.00 ppm pure 2,4-D, WAM40, and DMA4), measured for total length (±0.01 mm), and weighed for wet mass (±0.001 g).

Data analysis

All data were analyzed using SAS software, Ver 9.4 for Windows (SAS Institute). Prior to the use of parametric statistics, the assumption of normality was tested with a Shapiro–Wilk test, and the assumption of homoscedasticity was tested with Bartlett's test. When assumptions of normality and/or homoscedasticity were not met, appropriate data transformations were performed (e.g., log transformations). Parametric analyses were performed using the MIXED procedure, whereby best fit comparisons of equal and nonequal variance models were made using a log-likelihood ratio test. Based on these results, the most appropriate model was used for the analysis of data that met the basic assumptions for parametric analysis. Parametric analysis (analysis of variance) was followed by Dunnett's post hoc comparisons. For nonparametric analyses, we performed a Kruskal–Wallis test followed by a Dwass, Steel, Critchlow–Fligner multiple comparison analysis (Douglas and Michael 1991; Dietrich and Krieger 2009). Data are presented as means ± standard error of the mean (SEM; n = sample size). Significance was set at $p < 0.05$.

RESULTS

Experiment 1: Exposed adults, eggs, and larvae (WAM40)

We recorded no adult mortalities during the 15-d pre-exposure or 28-d exposure periods. Larvae exposed to WAM40 experienced >90% of their mortalities in the first 2 wk of exposure (Figure 1A). Larvae exposed to all 3 concentrations showed a decrease in survival compared with control over the 30-d exposure ($\chi^2 = 20.1183$, $df = 6$, $p = 0.0026$; Figure 1B). Previously published data (DeQuattro and Karasov 2016) from our laboratory illustrating a decrease in fathead minnow larval survival after exposure to DMA4 have been included in Figure 1 to allow a direct comparison of WAM40 and DMA4 exposure

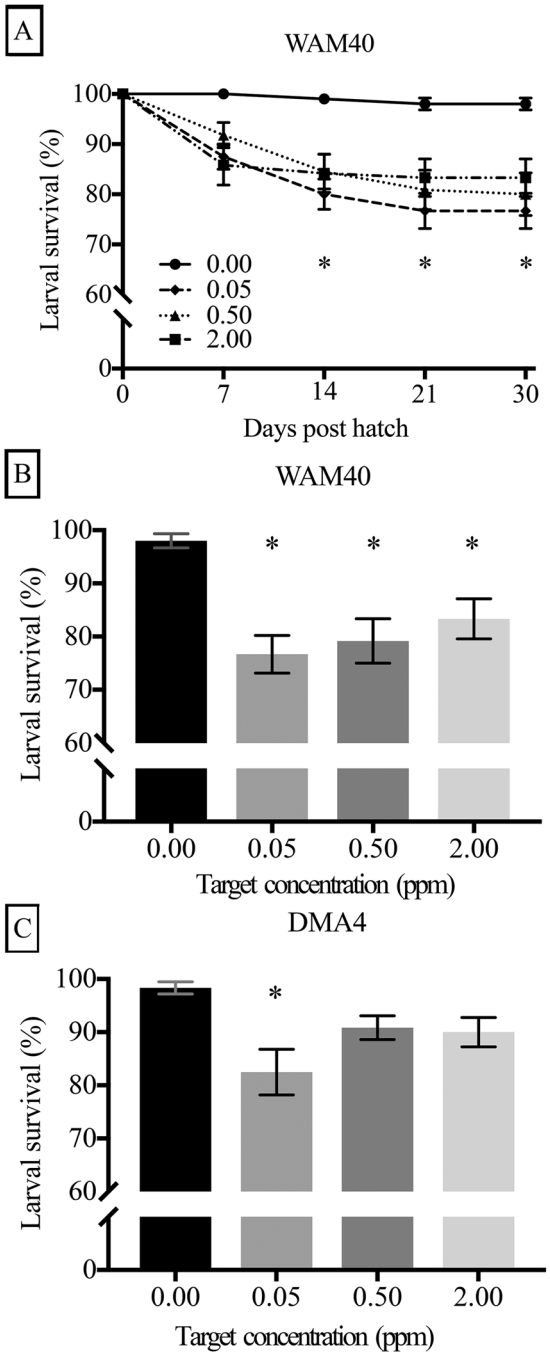

FIGURE 1: Survival of fathead minnow larvae in experiment 1, in which adults, eggs, and larvae were exposed to the 2,4-D formulations named within each panel. Survival (%) is shown as a function of days post hatch (**A**), or at 30 d post hatch (**B**). (**C**) Analogous data for comparison from DeQuattro and Karasov (2016). Data are mean ± standard error of the mean ($n = 10$–12 tanks in each treatment, each with 10 progeny from a pair of exposed adults). Asterisk indicates significantly different value ($p \leq 0.05$) compared with control by Dwass, Steel, Critchlow–Fligner comparison. WAM40 = Weedestroy® AM40; DMA4 = DMA® 4 IVM.

trials (Figure 1C). Surviving larvae exposed to WAM40 displayed an increase in both mass and total length at 2.00 ppm concentration (total length: $\chi^2 = 25.93$, $p = 0.0002$, mass: $\chi^2 = 29.46$, $p < 0.0001$; Figure 2A and B).

Experiment 2: Unexposed adults and exposed eggs and larvae (WAM40 and DMA4)

Larvae exposed to 0.50 and 2.00 ppm WAM40 showed a decrease in survival compared with control ($\chi^2 = 24.29$, $df = 6$, $p < 0.0001$; Figure 3A). We observed >90% of observed mortality during the first 2 wk of exposure (Figure 3B). Surviving larvae exposed to 2.00 ppm WAM40 displayed a significant increase in both mass and total length (total length: $F_{3,44} = 16.38$, $p < 0.0001$, mass: $\chi^2 = 24.75$, $p < 0.0001$; Figure 2C and D).

Larvae exposed to 0.50 ppm DMA4 showed a significant decrease in survival compared with control ($\chi^2 = 20.1183$, $df = 6$, $p = 0.0026$; Figure 3C). Again, we observed >90% of the mortality in the first 2 wk of exposure (Figure 3D). Surviving larvae exposed to 2.00 ppm DMA4 displayed a significant increase in total length compared with controls (total length: $F_{3,44} = 14.25$, $p = 0.0002$; Figure 2E and F).

Experiment 3: Unexposed adults and exposed eggs and larvae (WAM40, DMA4, pure 2,4-D)

Larvae exposed to 0.50 ppm pure 2,4-D, WAM40, and DMA4 showed a significant decrease in survival compared with controls ($\chi^2 = 30.42$, $df = 6$, $p < 0.0001$, $p < 0.0001$, $p < 0.0004$, respectively). Larvae exposed to 2.00 ppm DMA4 showed a significant decrease in survival compared with control ($\chi^2 = 30.42$, $df = 6$, $p < 0.0157$), whereas larvae exposed to 2.00 ppm WAM40 and pure 2,4-D did not show a significant difference in survival compared with controls ($p = 0.0557$, $p > 0.10$, respectively; Figure 4A). Again, we observed >90% of all observed mortality in the first 2 wk of exposure (Figure 4B). Surviving larvae exposed to 2.00 ppm DMA4 and WAM40 displayed a significant increase in total length and mass compared with controls (DMA4 total length: $\chi^2 = 52.62$, $p = 0.0160$, mass: $\chi^2 = 68.2$, $p < 0.0001$; WAM40 total length: $\chi^2 = 52.62$, $p = 0.0079$, mass: $\chi^2 = 68.2$, $p = 0.0044$; Figure 2G and H).

DISCUSSION

Overview

The results of the present study indicate that pure 2,4-D and the formulations WAM40 and DMA4 all significantly decreased fathead minnow larval survival at environmentally relevant concentrations within the allowed application concentrations permitted by the product labels (spot treatments ≤4 ppm and whole-lake treatments ≤2 ppm; US Environmental Protection Agency 2005). The 2,4-D amine herbicide formulations and pure 2,4-D caused significant and similar decreases in larval survival in fathead minnows exposed to concentrations as low as 0.50 ppm, suggesting that 2,4-D itself may be the causative agent in regard to larval survival. In all experiments, >90% of the larval mortality observed occurred within 14 dph. These results identify what may be a critical window of exposure for fathead minnow larvae when exposed to 2,4-D acid or its herbicide commercial formulations.

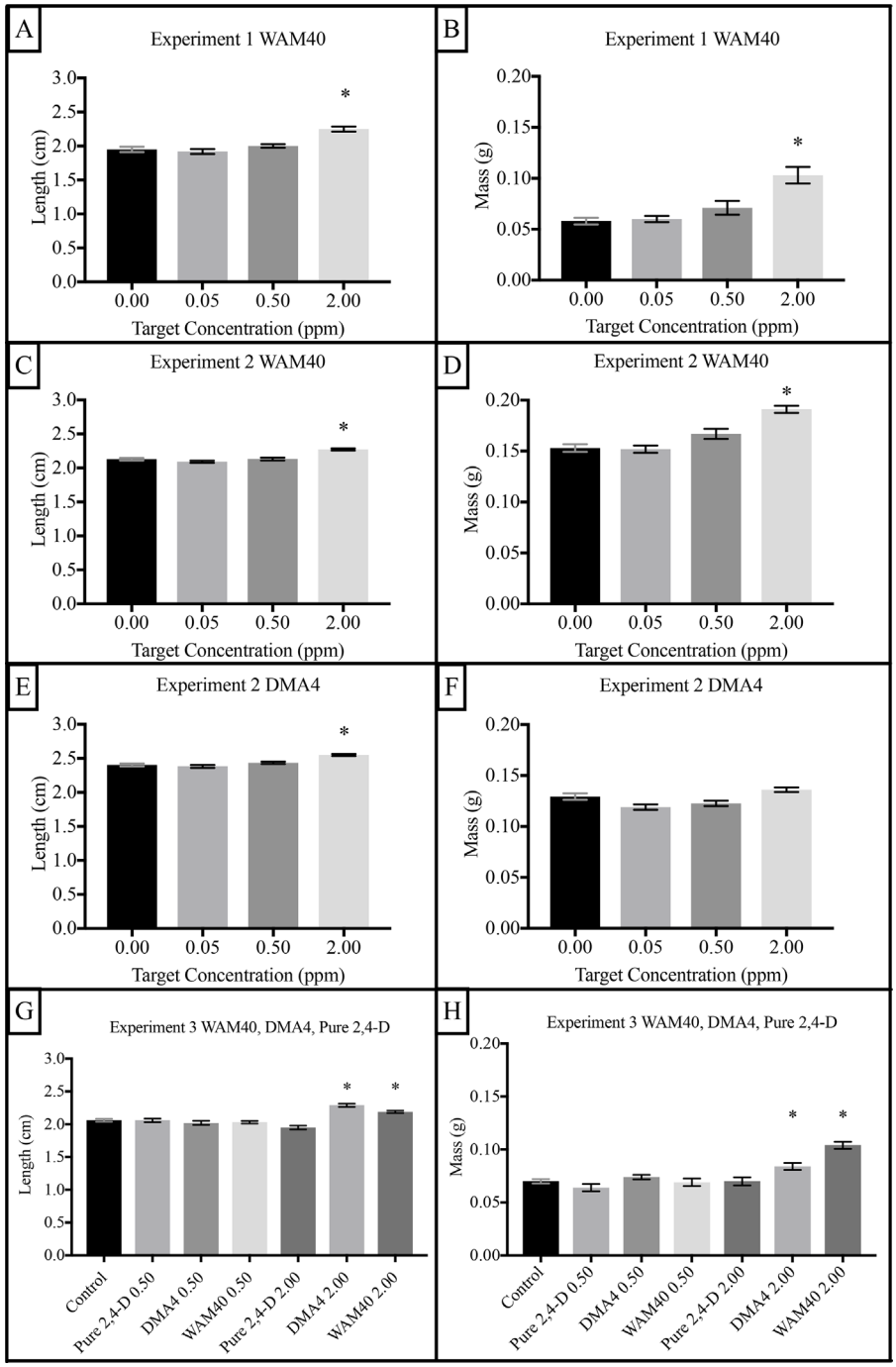

FIGURE 2: Length and mass of fathead minnow larvae raised in the different experiments, each of which was defined according to the particular formulation of 2,4-D, or pure compound that was used (identified in each panel), and according to life stages that were exposed (see Table 1 for description of each experiment). Values (mean ± standard error of the mean; $n = 10$–12 tanks in each treatment) of larvae total length (cm; **A, C, E, G**) and body mass (g wet mass; **B, D, F, H**) obtained at 30 d post hatch. Asterisk indicates a significantly different value ($p < 0.05$) compared with controls by Dwass, Steel, Critchlow–Fligner comparison. WAM40 = Weedestroy® AM40; DMA4 = DMA® 4 IVM; 2,4-D = 2,4-dichlorophenoxyacetic acid.

Pure 2,4-D versus 2,4-D herbicide formulations

Larvae exposed to pure 2,4-D and both formulations (WAM40 or DMA4) showed similar impacts on survival at 0.50 ppm. The results suggest that exposure to the active ingredient in these formulations, 2,4-D, is responsible for the negative survival impacts. In contrast, the literature has shown that pure 2,4-D compounds had no effect on survival at low, ecologically relevant concentrations on older juvenile and adult fathead minnows (US Environmental Protection Agency 2005; Coady et al. 2013; Vigário and Sabóia 2014). To our knowledge, no published studies have tested the impacts of environmentally

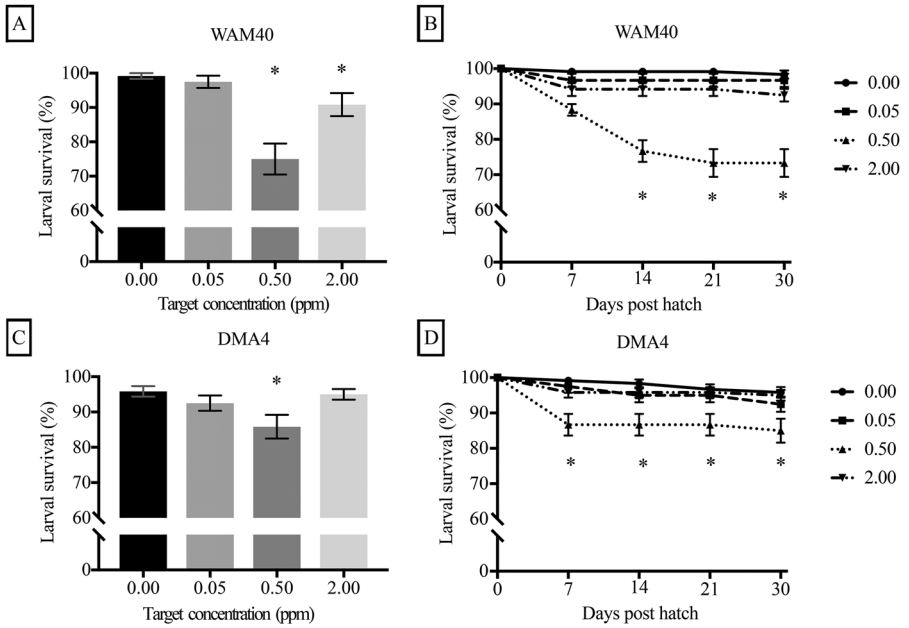

FIGURE 3: Survival of fathead minnow larvae in experiment 2, in which adults were unexposed, and eggs and larvae were exposed to the 2,4-D formulations named within each panel. Survival (%) is shown as a function of 30 d post hatch (**A** and **C**), or days post hatch (**B** and **D**). Data are mean ± standard error of the mean ($n = 12$ tanks in each treatment, each with 10 progeny from a pair of unexposed adults). Asterisk indicates significantly different value ($p \leq 0.05$) compared with control by Dwass, Steel, Critchlow–Fligner comparison. WAM40 = Weedestroy® AM40; DMA4 = DMA® 4 IVM.

relevant doses (0.00–2.00 ppm) of pure 2,4-D exposure on survival from fertilization to 14 dph in any fish species. Hence, our data, in conjunction with other published data, are consistent with pure 2,4-D being the causative toxic agent and fertilization to 14 dph being a sensitive life stage in fathead minnows.

The present study used commercial 2,4-D herbicide formulations that contained approximately 50% of the active ingredient and 50% ingredients that are labeled inert (as given on DMA4®IVM and Weedestroy®AM40 labels). Inert ingredients are protected as confidential business information (Northwest Coalition for Alternatives to Pesticides v. Browner 1996) or confidential by many governments (Organisation for Economic Co-operation and Development 1998), which makes it difficult to understand whether they play a role in toxicity. Some so-called inert ingredients that have been used in other 2,4-D formulations include solvents (such as triisopropanolamine and diethyleneglycol monoethyl), a silicone defoamer, and a proprietary surfactant, for example, polyglycol 26-2 (Oakes and Pollak 1999). Oakes and Pollak (1999) found that the proprietary surfactant polyglycol 26-2 in Tordon 75D® increased the toxicity of the active ingredients by either increasing the damage to cell membranes, increasing exposure of the active ingredients, or being toxic itself. However, in the present study with the DMA4 and WAM40 formulations, it was apparent that inert ingredients did not cause an increase in toxicity compared with the active ingredient, 2,4-D.

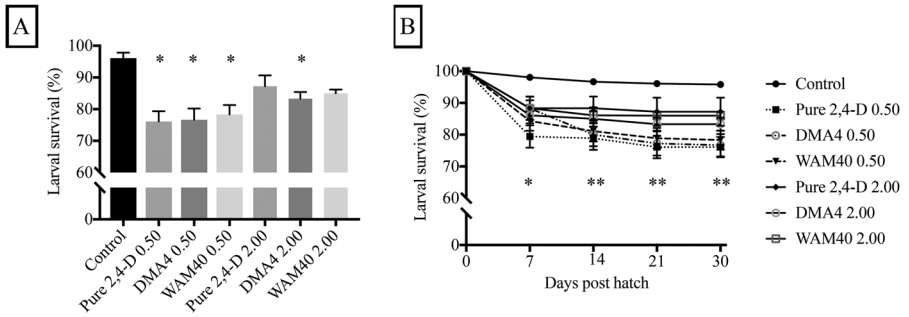

FIGURE 4: Survival of fathead minnow larvae in experiment 3, in which adults were unexposed, and eggs and larvae were exposed to either pure 2,4-D or DMA4 or WAM40. Survival (%) is shown as a function of 30 d post hatch (**A**), or days post hatch (**B**). Data are mean ± standard error of the mean ($n = 12$ tanks in each treatment, each with 15 progeny from a pair of unexposed adults). Asterisk indicates significantly different value ($p \leq 0.05$) compared with control by Dwass, Steel, Critchlow–Fligner comparison after the first week of exposure (0.50 ppm pure 2,4-D). Double asterisk indicates significantly different value ($p \leq 0.05$) compared with control by Dwass, Steel, Critchlow–Fligner comparison after the second week of exposure (0.50 ppm, DMA4, WAM40, and pure 2,4-D; 2.00 ppm DMA4). WAM40 = Weedestroy® AM40; DMA4 = DMA® 4 IVM; 2,4-D = 2,4-dichlorophenoxyacetic acid.

Critical window of exposure

The observation that >90% of larval mortality occurred during the first 14 dph, in all 3 experiments described, illustrates what may be a critical window during which exposure to 2,4-D, WAM40, or DMA4 seems to be impacting fathead minnow larval survival. Offspring exposed from fertilization to 30 dph to pure 2,4-D, WAM40, or DMA4 exhibited a significant depression of larval survival, regardless of parental exposure. Another experiment done in our laboratory showed no significant impacts on larval survival when only the parental generation was exposed (constant exposure through spawn) and larvae were hatched and raised to 30 dph unexposed (Supplemental Data, Figure S2). Moreover, neither WAM40 nor DMA4 seem to have immediate negative impacts on the survival of fathead minnow embryos (spawn to hatch) regardless of parental exposure (Supplemental Data, Figure S3; DeQuattro and Karasov 2016). These data taken together suggest that the larval stage, in particular from fertilization to 14 dph, is a critical window of exposure to 2,4-D.

The period between fertilization to 14 dph is considered to be a critical developmental stage in fish (Laale and Lerner 1981; Bentivegna and Piatkowski 1998; Sarikaya and Selvi 2005), and toxicant exposure can affect early developmental stages more than mature life stages (Lotufo and Fleeger 1997; Sarikaya and Selvi 2005; Mácová et al. 2008; Fairchild et al. 2009; Mensah et al. 2011). For example, swim-up rainbow trout, 10 to 14 d old, exposed to the 2,4-D herbicide amine formulation Weedar 64 showed significant adverse effects on growth parameters, whereas their juvenile counterparts were unaffected (Fairchild et al. 2009). Early developmental stages (embryo and larva) are typically more sensitive to xenobiotics because they have underdeveloped homeostatic mechanisms, immature immune systems, and underdeveloped organs (e.g., gills, liver, and kidney), which are important in the detoxification and elimination of xenobiotics (Bentivehna and Piatkowski 1998; Laale and Lerner 1981, McKim 1995; Mohammed 2013; Oikari et al. 2002). Further research is required to determine if embryonic exposure is required during the critical window of exposure to cause a decrease in larval survival as seen in the present study. Interestingly, exposure of germ cells in adults potentially makes larvae more susceptible to exposure during the critical window (fertilization to 14 dph) and may have compounding effects on fathead minnow larval survival (Baker et al. 2014; Galus et al. 2014; Schwindt et al. 2014; Xin et al. 2015; Xu et al. 2017). The present results suggest that future toxicant exposure studies on other native fish species should include toxicant exposure to early life stages.

Possible mechanisms of impacts on larval survival

We cannot for certain determine the mechanism by which 2,4-D and its herbicide formulations are exerting their low-dose impacts on larval survival. Low-dose responses are not uncommon in ecotoxicology (Bogers et al. 2006; Panter et al. 2010). The endocrine system is adapted to react to low concentrations of hormones (Tanaka et al. 1995), and low concentrations of toxicants can mimic endogenous hormones within the body, altering immune and metabolic responses (Tanaka et al. 1995; Inui et al. 1995; Panter et al. 2010; Song 2014). It is possible that low concentrations of 2,4-D may act as an endocrine active compound and affect nontarget sites, receptors, and/or pathways in nontarget species (Stebbins-Boaz et al. 2004; Xie et al. 2005; Hossain et al. 2008). Crucial endocrine systems are under development during early life stages (i.e., the hypothalamic–pituitary–interrenal, hypothalamic–pituitary–thyroid, hypothalamic–pituitary–gonadal axes; Inui et al. 1995; Bogers et al. 2006; Aslop and Vijayan 2009; Ankley and Johnson 2004). Adult fathead minnows exposed to DMA4 or WAM40 showed a decrease in tubercle count (DeQuattro and Karasov 2016), suggesting that 2,4-D or some other component of those formulations was acting as an antagonist of an androgen receptor–mediated pathway (Ankley et al. 2004). The androgen receptor has been shown to be active early in fish embryos and larvae (Hossain et al. 2008; Gorelick et al. 2008) and can affect a myriad of early life developmental and physiological pathways (Borg 1994). Given the role of androgens in early development, the established activity of the androgen receptor in fish embryos and larva, and previous findings that 2,4-D is endocrine active, it is possible that 2,4-D herbicides are exerting their impacts on larval survival via antagonism of the androgen receptor.

Another possible hypothesis for the impacts of 2,4-D on fathead minnow larval survival is that exposure to 2,4-D increases oxidative stress in multiple fish tissues, decreasing survival through an increase in the presence of reactive oxygen species (ROS) or interference with ROS deactivation pathways (Aoki 2001; Ozcan Oruc et al. 2004; Lushchak 2011; Atamaniuk et al. 2013; Tayeb et al. 2013). Fish exposed to 2,4-D have been shown to have increased oxidative stress in the kidneys, liver, and brain (Ozcan Oruc et al. 2004; Atamaniuk et al. 2013). Li et al. (2017) reported that zebrafish embryos exposed to 25 ppm 2,4-D showed a decreased hatch rate and survival, increased manifestation of pericardial edemas, and impacted regulation of key heart and oxidative stress biomarker genes. Although Li et al. (2017) used exposure concentrations above those of the present study and a different model aquatic vertebrate, their report provides insight into how chronic low-concentration exposures may be acting to induce the significant decreases in larval survival observed in the present study.

At this time, definitive conclusions cannot be made on the mechanism of action for decreased survival in fathead minnow larvae exposed to environmentally relevant concentrations of pure 2,4-D, WAM40, or DMA4 from post fertilization to 14 dph. Further research on the impacts of 2,4-D and commercial 2,4-D formulations at environmentally relevant concentrations is warranted. More specifically, exposures focused on the early life stage of fishes that monitor the impacts of exposure on both ROS biomarkers and androgen receptor expression profile could help to elucidate the mechanism of 2,4-D toxicity.

Increase in growth

Larvae exposed to 2.00 ppm WAM40 and DMA4 showed significant increases in total length and/or mass compared with control. The current findings contradict those of Menezes et al.

(2015); in their study, juvenile silver catfish showed a decrease in both body weight and total length when exposed to the 2.00 ppm 2,4-D herbicide formulation U 46 D-fluid. However, Menezes et al. (2015) used a different life stage, a different species, and a different formulation. Any or all of these differences may account for the different results in the present study (US Environmental Protection Agency 2005). An increase in resources or lack of competition is not the likely cause of increase in size, because we did not observe significant increases in length or mass in larval fish exposed to 0.50 ppm, the treatment with the largest reduction in survival. Furthermore, the 2,4-D compound itself seems not likely to be the cause of the observed increases in size, because larval fish exposed to 2.00 ppm pure 2,4-D did not show an increase in either length or mass compared with controls. Thus, the observed results could be due to other compounds in the formulations acting directly or through an interaction between the labeled inert ingredients and 2,4-D. These formulations contain up to 53% of putatively inert ingredients that manufacturers do not specify. Currently, no conclusion on the mechanism of action can be made as to why larvae exposed to 2.00 ppm DMA4 or WAM40 post fertilization showed an increase in both total length and mass.

Management implications

At the current concentrations permitted by the USEPA for 2,4-D amine herbicide formulations, the application of WAM40 and DMA4 to aquatic ecosystems likely decreases fathead minnow larval survival and recruitment and could impact other native fishes similarly. In the present study, exposure of fathead minnows to pure 2,4-D and 2,4-D amine formulations at concentrations <2.00 ppm during fertilization to 14 dph caused the most significant decrease in larval survival. These impacts on fishes are most likely realized when herbicide application coincides with the breeding cycles and recruitment periods. However, given previous studies illustrating a potentially long latency period (i.e., up to 93 d; Nault et al. 2014) of 2,4-D in the water column, risk periods to fish may range from 21 d to months after application dates (US Environmental Protection Agency 2005). Furthermore, exposure of the germ cells in adults potentially makes the larvae more susceptible to a broader range of concentrations (0.05–2.00 ppm; DeQuattro and Karasov 2016). These findings suggest that permitting these herbicide formulations, based solely on data provided using juvenile and adult stages exposed to the pure 2,4-D acid and amine forms, should be re-evaluated.

CONCLUSIONS

The present study showed that exposure of fathead minnow larvae to pure 2,4-D, DMA4, and WAM40 significantly decreased larval survival and demonstrated that the complete formulations, WAM40 and DMA4, did not increase toxicity compared with the active ingredient 2,4-D. The critical window of exposure for pure 2,4-D and both formulations was from fertilization to 14 dph. Significant impacts on survival were detected at concentrations below USEPA-permitted application limits. Furthermore, it seems that 2,4-D is the causative agent decreasing larval survival. Our results can be used to guide new toxicological studies using 2,4-D in the future. The permitted use of 2,4-D herbicide formulations in aquatic environments warrants caution, and a re-evaluation is needed for permitting regulations.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4209.

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Data availability—To request data please contact Gavin Dehnert at dehnert2@wisc.edu.

REFERENCES

Ankley GT, Johnson RD. 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *Inst Lab Anim Res J* 45:469–483.

Ankley GT, Villeneuve DL. 2006. The fathead minnow in aquatic toxicology: Past, present and future. *Aquat Toxicol* 78:91–102.

Ankley GT, Defoe DL, Kahl MD, Jensen KM, Makynen EA, Miracle A, Hartig P, Gray LE, Cardon M, Wilson V. 2004. Evaluation of the model anti-androgen flutamide for assessing the mechanistic basis of responses to an androgen in the fathead minnow (*Pimephales promelas*). *Environ Sci Technol* 38:6322–6327.

Aoki Y. 2001. Polychlorinated biphenyls, polychloronated dibenzo-p-dioxins, and polychlorinated dibenzofurans as endocrine disrupters—What we have learned from yusho disease. *Environ Res* 86:2–11.

Aslop D, Vijayan M. 2009. Molecular programming of the corticosteroid stress axis during zebrafish development. *Comp Biochem Physiol* 153:49–54.

Atamaniuk TM, Kubrak OI, Storey KB, Lushchak VI. 2013. Oxidative stress as a mechanism for toxicity of 2,4-dichlorophenoxyacetic acid (2,4-D): Studies with goldfish gills. *Ecotoxicology* 22:1498–1508.

Baker TR, King-Heiden TC, Peterson RE, Heideman. 2014. Dioxin induction of transgenerational inheritance of disease in zebrafish. *Mol Cell Endocrinol* 398:36–41.

Barbieri E. 2009. Effect of 2,4-D herbicide (2,4-dichlorophenoxyacetic acid) on oxygen consumption and ammonium excretion of juveniles of *Geophagus brasiliensis* (Quoy & Gaimard, 1824) (Osteichthyes, Cichlidae). *Ecotoxicology* 18:55–60.

Bentivegna CS, Piatkowski T. 1998. Effects of tributyltin on medaka (*Oryzias latipes*) embryos at different stages of development. *Aquat Toxicol* 44:117–128.

Bogers R, Vries-Buitenweg S, Gils M, Baltussen E, Hargreaves A, Waart B, Roode D, Legler J, Murk A. 2006. Development of chronic tests for endocrine active chemicals: Part 2: An extended fish early-life stage test with an androgenic chemical in the fathead minnow (*Pimephales promelas*). *Aquat Toxicol* 80:119–130.

Borg B. 1994. Androgens in teleost fishes. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 109:219–245.

Coady K, Marino T, Thomas J, Sosinski L, Neal B, Hammond L. 2013. An evaluation of 2,4-dichlorophenoxyacetic acid in the Amphibian Metamorphosis Assay and the Fish Short Term Reproduction Assay. *Ecotoxicol Environ Saf* 90:143–150.

Cox C, Surgan M. 2006. Unidentified inert ingredients in pesticides: Implications for human and environmental health. *Environ Health Perspect* 114:1803–1806.

DeQuattro Z, Karasov W. 2016. Impacts of 2,4-D aquatic herbicide formulations on reproduction and development of the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 35:1478–1488.

Dietrich D, Krieger H. 2009. *Histological Analysis of Endocrine Disruptive Effects in Small Laboratory Fish*. John Wiley & Sons, Hoboken, NJ, USA.

Douglas C, Michael F. 1991. On distribution-free multiple comparisons in the one-way analysis of variance. *Commun Stat Theor Methods* 20:127–139.

Fairchild J, Feltz K, Allert A, Sappington L, Nelson K, Valle J. 2009. An ecological risk assessment of the exposure and effects of 2,4-D acid to rainbow trout (*Onchorhyncus mykiss*). *Environ Contam Toxicol* 56:754–760.

Galus M, Rangarajan S, Lai A, Balshine S, Wilson JY. 2014. Effects of chronic, parental pharmaceutical exposure on zebrafish (*Danio rerio*) offspring. *Aquat Toxicol* 151:124–134.

Gorelick DA, Watson W, Halpern ME. 2008. Androgen receptor gene expression in the developing and adult zebrafish brain. *Dev Dyn* 237:2987–2995.

Grossmann K. 2003. Mediation of herbicide effects by hormone interactions. *J Plant Growth Regul* 22:109–122.

Hossain MS, Larsson A, Scherbak N, Olsson P-E., Orban L. 2008. Zebrafish androgen receptor: Isolation, molecular, and biochemical characterization. *Biol Reprod* 78:361–369.

Inui Y, Yamano K, Miwa S. 1995. The role of thyroid hormone in tissue development in metamorphosing flounder. *Aquaculture* 135:87–98.

Kahn RG, Pearson DE, Dick EJ. 2004. Comparison of standard length, fork length, and total length for measuring west coast marine fishes. *Mar Fish Rev* 66:31–33.

Laale HW, Lerner W. 1981. Teratology and early fish development. *Am Zool* 21:517–533.

Li K, Wu J-Q., Jiang L-L., Shen L-Z., Li J-Y., He Z-H., Wei P, Lv Z, He M-F. 2017. Developmental toxicity of 2,4-dichlorophenoxyacetic acid in zebrafish embryos. *Chemosphere* 171:40–48.

Lotufo G, Fleeger J. 1997. Effects of sediment-associated phenanthrene on survival, development and reproduction of two species of meiobenthic copepods. *Mar Ecol Prog Ser* 151:91–102.

Lushchak VI. 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol* 101:13–30.

Mácová S, Doleželová P, Pištěková V, Svobodová Z, Bedáňová I, Voslářová E. 2008. Comparison of acute toxicity of 2-phenoxyethanol and clove oil to juvenile and embryonic stages of *Danio rerio*. *Neuroendocrinol Lett* 29:680–684.

McKim JM. 1995. Early life stage toxicity tests. In Rand GM, ed, *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate and Risk Assessment*. Taylor & Francis, Washington, DC, USA.

Menezes C, Fonseca M, Leitemperger J, Pretto A, Moraes B, Murussi C, Baldisserotto B, Loro V. 2015. Commercial formulation containing 2,4-D affects biochemical parameters and morphological indices of silver catfish exposed for 90 days. *Fish Physiol Biochem* 41:323–330.

Mensah P, Muller W, Palmer C. 2011. Acute toxicity of Roundup® herbicide to three life stages of the freshwater shrimp *Caridina nilotica* (Decapoda: Atyidae). *Phys Chem Earth* 36:905–909.

Mesnage R, Defarge N, Soiroux de Vendomois J, Seralini GE. 2014. Major pesticides are more toxic to human cells than their declared active principles. *Biomed Res Int*. DOI:10.1155/2014/179691

Mohammed A. 2013. Why are early life stages of aquatic organisms more sensitive to toxicants than adults? In Gowder SJT, ed, *New Insights into Toxicity and Drug Testing*. InTech. DOI: 10.5772/55187. [cited 2017 February 15]. Available from: http://www.intechopen.com/books/new-insights-into-toxicity-and-drug-testing/why-are-early-life-stages-of-aquatic-organisms-more-sensitive-to-toxicants-than-adults-

Nault ME, Netherland MD, Mikulyuk A, Skogerboe JG, Asplund T, Hauxwell J, Toshner P. 2014. Efficacy, selectivity, and herbicide concentrations following a whole-lake 2,4-D application targeting Eurasian watermilfoil in two adjacent northern Wisconsin lakes. *Lake Reservoir Manage* 30:1–10.

Northwest Coalition for Alternatives to Pesticides v. Browner. 1996. Case No. 94-1100, US District Court for the District of Columbia, Washington, DC, USA.

Oakes DJ, Pollak JK. 1999. Effects of a herbicide formulation, Tordon 75D®, and its individual components on the oxidative functions of mitochondria. *Toxicology* 136:41–52.

Oakes DJ, Pollak JK. 2000. The in vitro evaluation of the toxicities of three related herbicide formulations containing ester derivatives of 2,4,5-T and 2,4-D using sub-mitochondrial particles. *Toxicology* 151:1–9.

Organisation for Economic Co-operation and Development. 1998. OECD governments' approaches to the protection of proprietary rights and confidential business information in pesticide registration. [cited 2018 February 3]. Available from: http://www.olis.oecd.org/olis/1998doc.nsf/LinkTo/env-mc-chem(98)20

Oikari A, Fragoso N, Leppänen H, Chan T, Hodson PV. 2002. Bioavailability to juvenile rainbow trout (*Oncorynchus mykiss*) of retene and other mixed-function oxygenase-active compounds from sediments. *Environ Toxicol Chem* 21:121–1288.

Ozcan Oruc E, Sevgiler Y, Uner N. 2004. Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. *Comp Biochem Physiol C Toxicol Pharmacol* 137:43–51.

Panter GH, Hutchinson TH, Hurd KS, Bamforth J, Stanley RD, Wheeler JR, Tyler CR. 2010. Effects of a weak oestrogenic active chemical (4-tert-pentylphenol) on pair-breeding and F1 development in the fathead minnow (*Pimephales promelas*). *Aquat Toxicol* 97:314–323.

Pérez GL, Vera MS, Miranda L. 2011. Effects of herbicide glyphosate and glyphosate-based formulations on aquatic ecosystems. In Kortekamp A, ed, *Herbicides and Environment*. InTech, pp 343–368.

Ritter W. 1990. Pesticide contamination of ground water in the United States. *J Environ Sci Health* 25:1–29.

Ruiz de Arcaute C, Soloneski S, Larramendy M. 2016. Toxic and genotoxic effects of the 2,4-dichlorophenoxyaceticacid (2,4-D)-based herbicide on the neotropical fish *Cnesterodon decemmaculatus*. *Ecotocicol Environ Saf* 128:222–229.

Sarikaya R, Selvi M. 2005. Investigation of acute toxicity of (2,4-dichlorophenoxy) acetic acid (2,4-D) herbicide on larvae and adult Nile tilapia (*Oreochromis niloticus* L.). *Environ Toxicol Pharmacol* 20:264–268.

Schwindt A, Winkelman, D Keteles, K, Murphy M, Vajda A. 2014. An environmental oestrogen disrupts fish population dynamics through direct and transgenerational effects on survival and fecundity. *J Appl Ecol* 51:582–591.

Song Y. 2014. Insight into the mode of action of 2, 4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. *J Integr Plant Biol* 56:106–113.

Stebbins-Boaz B, Fortner K, Frazier J, Piluso S, Pullen S, Rasar M, Reid W, Sinclair K, Winger E. 2004. Oocyte maturation in *Xenopus laevis* is blocked by the hormonal herbicide, 2,4-dichlorophenoxy acetic acid. *Mol Reprod Dev* 67:233–242.

Tanaka M, Tanangonan J, Tagawa M, de Jesus E, Nishida H, Isaka M, Kimura R, Hirano T. 1995. Development of the pituitary, thyroid and interrenal glands and applications of endocrinology to the improved rearing of marine fish larvae. *Aquaculture* 135:111–126.

Tayeb W, Nakbi A, Cheraief I, Miled A, Hammami M. 2013. Alteration of lipid status and lipid metabolism, induction of oxidative stress and lipid peroxidation by 2,4-dichlorophenoxyacetic herbicide in rat liver. *Toxicol Mech Methods* 23:449–458.

US Environmental Protection Agency. 2005. Reregistration eligibility decision for 2,4-D. Washington, DC. [cited 2017 January 5]. Available from: http://www.epa.gov/pesticides/pestsales/01pestsales/table_of_contents2001.htm#pdfreport

Vigário A, Sabóia S. 2014. Effects of the 2,4-D herbicide on gills epithelia and liver of the fish *Poecilia vivipara*. *Pesq Vet Bras* 34:523–528.

Von Westernhagen H. 1988. 4 sublethal effects of pollutants on fish eggs and larvae. *Fish Physiol* 11A:253–346.

Xie L, Thrippleton K, Irwin M, Siemering G, Mekebri A, Crane D, Berry K, Schlenk D. 2005. Evaluation of estrogenic activities of aquatic herbicides and surfactants using a rainbow trout vitellogenin assay. *Toxicol Sci* 87:391–398.

Xin F, Susiarjo M, Bartololmei M. 2015. Multigenerational and transgenerational effects of endocrine disrupting chemicals: A role for altered epigenetic regulation? *Semin Cell Dev Biol* 43:66–75.

Xu Q, Wu D, Dang Y, Yu L, Liu C, Wang J. 2017. Reproduction impairment and endocrine disruption in adult zebrafish (*Danio rerio*) after waterborne exposure to TBOEP. *Aquat Toxicol* 182:163–171.

